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PRINCIPAL INVESTIGATOR: David B. Vaught, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University
Nashville, TN 37232

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David B. Vaught

E-Mail: david.b.vaught@vanderbilt.edu

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Vanderbilt University
777 PRB
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14. ABSTRACT

Studies have shown that ErbB3 and ErbB4 (the neuregulin receptors) are required for growth and survival of the two major luminal mammary epithelial cell types, the ductal luminal mammary epithelium, and the milk-producing alveolar mammary epithelium. However, as both ErbB3 and ErbB4 are capable of binding to neuregulins and heterodimerizing with other ErbB family members, there is evidence that ErbB4 may compensate for loss of ErbB3, and vice versa making it likely that the neuregulin receptors drive cell growth and survival of breast cancers derived from the luminal breast epithelium. Our early studies demonstrate loss of ErbB3 expansion of the alveolar epithelium during pregnancy, decreased expression of genes encoding milk proteins, and decreased milk delivery to nursing pups. This is similar to what is seen in ErbB4-deficient mammary glands. We are currently studying the ErbB3-dependnet molecular mechanisms required for expansion of the alveolar epithelium during pregnancy, and have generated mice that lack ErbB3 and ErbB4 in the alveolar mammary epithelium for specific analysis of neuregulin loss in mammary morphogenesis, lactational differentiation, and breast cancer development.

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Breast Cancer, ErbB3, ErbB4, Neuregulin. Lactation, Pregnancy

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INTRODUCTION

ErbB3 and ErbB4 are activated by neuregulins (NRGs), a family of related ligands including NRG-1, -2, -3, and -4. The ErbB3 and ErbB4 receptors are required for growth and survival of the ductal and alveolar luminal epithelium, respectively. Recent work has further shown that ErbB3 is required to maintain the balance of differentiated epithelial cell types in the mammary gland, consistent with the fact the ErbB3 expression is highest in the ductal luminal epithelium and in luminal progenitor populations of the mouse and human mammary glands (1). ErbB4 is also required for growth in the luminal mammary epithelium, but in the alveolar luminal population that undergoes massive expansion during pregnancy, producing milk during lactation. Loss of ErbB4 prevents growth of the alveolar epithelium due to loss of STAT5A signaling. ErbB4 interacts directly with STAT5A and Jak2, making ErbB4 part of the mitogenic prolactin-Jak2-STAT5 signaling cascade (2). ErbB3 is expressed at highest levels in luminal breast cancers as compared to other molecular breast cancer subtypes, as shown by our recent analysis of several clinical breast cancer expression datasets. Others have demonstrated that ErbB4 expression is highest in luminal breast cancers as well. Human luminal breast cancer cell lines also express higher levels of ErbB3 and ErbB4 than cell lines derived from other molecular breast cancer subtypes (1). ErbB3 targeting decreases tumor growth in mouse and human models of luminal breast cancer (3). We have recently demonstrated that loss of ErbB3 decreases cell growth and survival in two models of spontaneous breast cancers classified as luminal B based on expression data (3). Loss of ErbB3 delayed tumor latency in one model and prevented tumor formation in the other (4). Although it has been reported that loss of ErbB4 did not affect formation of MMTV-Neu tumors, we and others have reported that expression of a specific splice isoform of ErbB4 referred to as ErbB4-Cyt2 increased growth of breast cells, and we demonstrated ErbB4-Cyt2 caused hyperproliferation in the mouse mammary epithelium (5). Thus using our newly developed mouse model we will be able to delineate neuregulin signaling on mammary gland development and tumor formation in the luminal mammary epithelium as achieved through double tissue specific gene targeting of ErbB3 and ErbB4.

BODY

Task 1: To determine the role of NRG signaling in physiological expansion of the luminal mouse mammary epithelium and its long-term effects on epithelial homeostasis. (Months 1-18)
1A)*. Is NRG signaling required for expansion of the alveolar luminal population during pregnancy?*
Begin breeding to cross ErbB3$^{WAP\text{-}KO}$ with ErbB4$^{WAP\text{-}KO}$ mice to generate E3/E4$^{WAP\text{-}KO}$ animals. Animals are on the same background already thus we expect to have isogenic populations of E3/E4$^{WAP\text{-}KO}$ animals within the first nine months to one year. (Months 1-12)
Once isogenic animals are obtained, at six weeks of age dams will undergo a single round of pregnancy and have their mammary glands harvested at 9.5, 13.5 and 17.5 dpc and lactation day 1 (L1) and L10. Nulliparous mice will serve as controls collected at the same time points as uniparous mice. Mammary gland tissue will be collected and either snap-frozen in liquid nitrogen for protein (lactogenic markers, β-casein and α-lactalbumin),RNA and qRT-PCR analysis (neuregulins, epiregulin, and betacellin), processed for whole mount analysis (epithelial-to-adipose ratio), or processed for FFPE IHC assessment to analyze proliferation, apoptosis, or key targets of ErbB3/ErbB4 signaling (P-Akt, P-STAT5, P-MAPKp44/42, and P-Jak2). (Months 12-18)

Task1 Research Accomplishments
Following regulatory approval for animal studies from the Vanderbilt Universtiy Institutional Animal Care and Use Committee (IACUC), breeding of ErbB3$^{Wap\text{-}KO}$ to ErbB4$^{FL/FL}$ animals. Resulting heterozygous progeny for ErbB3, ErbB4, and Cre were crossed together resulting in various genotypes for the progeny. Those offspring that were homozygous for ErbB3 or ErbB4 and heterozygous for the other gene plus positive for Cre were used as breeders to generate the next generation that resulted in homozygous ErbB3, ErbB4, Cre progeny that were then used as breeders to build up the mouse line for ErbB3/ErbB4$^{WAP\text{-}KO}$. Subsequent matings were set-up to generate ErbB3$^{WAP\text{-}KO}$ and ErbB4$^{WAP\text{-}KO}$ mouse lines for comparison studies between single and double tissue specific receptor knockout.

Figure 1. Genotypes from ErbB3KO WapCre animals crossed to ErbB4KO animals

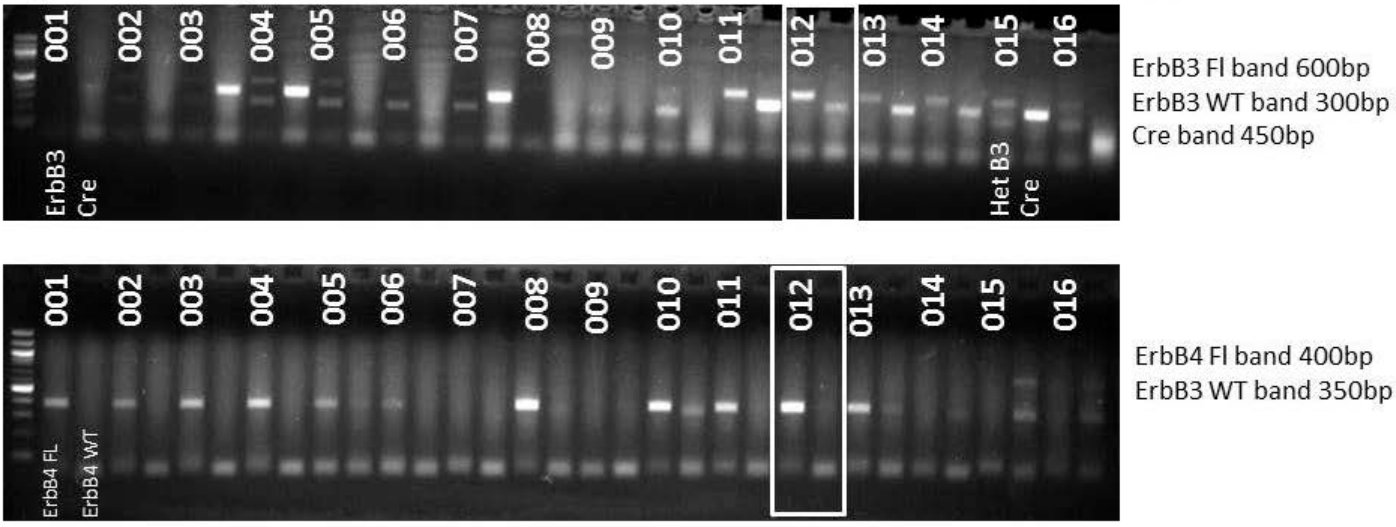

Figure 1. ErbB3 ErbB4 Cre Gentoyping Results. Progeny were screened for ErbB3 and ErbB4 loss as well as presence of Cre via PCR. ErbB3 PCR products were loaded together with top band corresponding to floxed ErbB3 and bottom band representing wildtype ErbB3 and Cre PCR products loaded in the following lane. ErbB4 PCR products were loaded next to each other with ErbB4 floxed products in the first lane followed by wildtype ErbB4. Animal number 12 demonstrates a ErbB3$^{Fl/FL}$ ErbB4$^{Fl/FL}$ Cre positive animal. Marker on far left of each gel is 100bp marker with bright bands corresponding to 500bp and 1000bp.

1*B)*. *Does NRG signaling during pregnancy effect epithelial proliferation, survival, and gene expression events in the aged mammary gland?* (Months 12-30)
ErbB3/ErbB4$^{WAP\text{-}KO}$ animals (plus single knockouts) will undergo a single round of pregnancy, lactation, and involution and their mammary glands will be harvested at post-lactational day 21 to confirm complete involution. Mammary glands from corresponding uniparous animals will be harvested at 12 months of age, as will mammary glands from 12-month old nulliparous mice. Glands collected at day 21 post-lactation and collected at 12 months of age will be either snap-frozen in liquid nitrogen for protein, RNA and qRT-PCR analysis, processed for whole mount analysis, or processed for FFPE IHC assessment to measure proliferation, cell death, epithelial density, and signaling through the ErbB3/ErbB4 pathways (PI3K, MAPK, and Jak2/STAT5). (Months 12-24)
Single cell mammary gland disaggregates will be analyzed by flow cytometry to quantify the luminal subpopulations within uniparous and nulliparous mammary glands from each group, using previously validated cell surface markers. (Months 12-24)
Gene expression changes in the aged multiparous and nulliparous mammary epithelium will be analyzed using laser capture microdissection of breast tissues collected. These individual tissues will be compared using SABiosceinces custom PCR arrays to analyze mammary stroma for each group of uniparous and multiparous mice based on published gene expression signatures corresponding to uniparous female mice mammary glands at 60 days post involution. (Months 24-30)

Task 2: To determine the role of NRG signaling in oncogene-driven expansion of the luminal mouse mammary epithelium. (Months 12-36)
2A). *Is NRG signaling required for oncogene-induced hyperplasia of the alveolar luminal population during pregnancy?*
Begin breeding of ErbB3$^{WAP\text{-}KO}$,ErbB4$^{WAP\text{-}KO}$, and ErbB3/ErbB4$^{WAP\text{-}KO}$ animals with p110* spontaneous mouse model of breast cancer. Mammary glands will be harvested from nulliparous mice and from mice at 9.5, 13.5 and 17.5 dpc. for analysis by immunoblot of ErbB3 and ErbB4 expression as well as for whole mount analysis and whole mount hematoxylin staining to view alveolar expansion as well as proliferation and cell death of the alveolar epithelium will be measured by Ki67 and cleaved caspase-3 staining. (Months 12-24)
2B). *Does NRG signaling during pregnancy affect tumorigenesis in aged mammary glands?*
Mammary glands from p110* ErbB3$^{WAP\text{-}KO}$, p110* ErbB4$^{WAP\text{-}KO}$ and p110* ErbB3/ErbB4$^{WAP\text{-}KO}$ animals that under went a single round of pregnancy were harvested at post-lactational day 21 to confirm complete involution. Mammary glands from corresponding uniparous animals will be harvested at 12 months of age, as will mammary glands from 12-month old nulliparous mice. Glands collected at day 21 post-lactation and collected at 12 months of age will be either snap-frozen in liquid nitrogen for protein, RNA and qRT-PCR analysis, processed for whole mount analysis, or processed for FFPE IHC assessment to measure proliferation, cell death, epithelial density, and signaling through the ErbB3/ErbB4 pathways (PI3K, MAPK, and Jak2/STAT5). (Months 18-36)
2C). p110* ErbB3$^{WAP\text{-}KO}$, p110* ErbB4$^{WAP\text{-}KO}$, and p110* ErbB3/ErbB4$^{WAP\text{-}KO}$ animals will be palpated weekly for tumor formation. Further, tumor latency, rate of tumor growth, number of tumors per mouse and lung metastases will be scored for analysis of neuregulin signaling. (Months 18-30)
2D). Tumor lineage markers will be assessed by immunohistochemical detection and flow cytometry of cytokeratins (CK)-8, CK14, CK5, estrogen receptor, and ErbB2 in isolated tumor cells. (Months 30-36)

Task 1B and Task 2 are proposed for the second year of research; thusly these tasks will be reported on the second annual report.

KEY RESEARCH ACCOMPLISHMENTS

ErbB3$^{WAP\text{-}KO}$ knockout mouse line was established and used to cross with ErbB4$^{FL/FL}$ mouse line to establish the ErbB3/ErbB4$^{WAP\text{-}KO}$ knockout mouse line. Also have independently established ErbB4$^{WAP\text{-}KO}$ knockout mouse line for comparison studies outlined in research proposal for year two research.

REPORTABLE OUTCOMES

Poster Presentations:

Neuregulin signaling in development and transformation of the luminal breast epithelium
David Vaught, Donna Hicks, and Rebecca S. Cook
Advances in Breast Cancer Research, October 2013, San Diego, CA

Neuregulin signaling in development and transformation of the luminal breast epithelium
David Vaught, Donna Hicks, and Rebecca S. Cook
13th Annual Host Tumor Interactions Program and Department of Cancer Biology Retreat
Vanderbilt University, November 2013, Nashville, TN

Animal Models:

ErbB3$^{WAP\text{-}KO}$ knockout mouse line generated
ErbB4$^{WAP\text{-}KO}$ knockout mouse line generated
ErbB3/ErbB4$^{WAP\text{-}KO}$ knockout mouse line generated

CONCLUSION

Of the four members of the ErbB family of receptor tyrosine kinases (RTKs) EGFR and ErbB2 are often overexpressed/dysregulated in human breast cancers and studies have demonstrated that ErbB2/ErbB3 heterodimers are the most potently oncogenic heterodimeric pair within the family. Beyond the ErbB2-amplified subtype of breast cancers, there is little known regarding the role of ErbB3 and even less is known about ErbB4 in human breast cancers. Despite this, research from our lab and others has demonstrated that developmental processes within the luminal compartment of the breast epithelium, like luminal specification, cell proliferation, and cell survival, require ErbB3 and ErbB4 (the neuregulin receptors). Cell growth and survival are two processes central to the formation of every cancer. Given that ErbB3 and ErbB4 are uniquely required within the luminal breast epithelium during phases of extreme physiological proliferation (i.e., puberty and pregnancy, respectively) we hypothesize that elimination of NRG signaling by combined loss of ErbB3 and ErbB4 would prevent expansion of the mammary epithelium in response to physiological cues (i.e. pregnancy) and pathological cues (oncogene expression).

To fully address the roles of ErbB3 and ErbB4 in the expansion of the mammary epithelium, tissue specific receptor knockout mice had to be developed. This first progress report has highlighted the breeding strategy and displayed genotyping results corresponding to the development of the ErbB3/ErbB4$^{WAP\text{-}KO}$ knockout mouse that is the foundation for subsequent studies. In addition, we have generated single knockout mice of ErbB3$^{WAP\text{-}KO}$ and ErbB4$^{WAP\text{-}KO}$ to investigate the role of each receptor in regulation of mammary epithelium expansion as compensatory mechanisms are investigated in conjunction with complete loss of neuregulin signaling.

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